

## CLAIMS

1. A screening method of molecules capable of generating the alteration of a target intracellular parameter, said alteration being converted into a proportional variation in the intracellular concentration of the  $\text{Ca}^{2+}$  ion, detected by means of a  $\text{Ca}^{2+}$ -sensitive recombinant protein probe, comprising the following phases:
- a) construction of an expression vector containing the sequence encoding said probe, said sequence being characterized in that it comprises sequences encoding at least one  $\text{Ca}^{2+}$ -sensitive photo-protein, and at least one cellular effector or a signal sequence, condensed together;
  - b) transfection of at least one cellular line of a mammal with said vector containing the  $\text{Ca}^{2+}$ -sensitive recombinant protein probe;
  - c) activation of said  $\text{Ca}^{2+}$ -sensitive photo-protein by the addition of a prosthetic group to the cellular line expressing said recombinant protein probe;
  - d) administration of the molecule to be tested to the cellular line expressing said recombinant protein probe;
  - e) detection of the emission of photons on the part of the  $\text{Ca}^{2+}$ -sensitive photo-protein expressed in the cellular line and evaluate the amount of activation or inhibition exerted by the tested molecule, on the basis of a ratio between the cps value obtained and the maximum

value of cps registered under conditions of maximum stimulation of the cellular line.

2. The method according to claim 1, wherein said  $\text{Ca}^{2+}$ -sensitive photo-protein is aequorin.

5 3. The method according to claim 1 and 2, wherein said prosthetic group is celenterazine.

4. The method according to claims 1 to 3, wherein said alteration of an intracellular parameter is selected from the group which comprises variation in the concentration 10 of a second messenger, translocation to the membrane or activation/inactivation state of a cellular effector.

5. The method according to claim 4, wherein said second messenger is selected from the group which comprises cyclic nucleotides, adenosine nucleotides, diacylglycerol, 15  $\text{Ca}^{2+}$  and inositol 1,4,5-triphosphate.

6. The method according to claim 4, wherein said cellular effector is selected from the group which consists of ionic channel, regulating protein, cellular membrane receptor.

20 7. The method according to claim 6, wherein said ionic channel is selected from the group which comprises voltage dependent  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  channel-receptors.

8. The method according to claim 6, wherein said regulating protein is selected from the group which comprises 25 protein-kinase, phosphatase, adenylate cyclase, proteins

that link plasmatic membrane receptors, proteins that interact with plasmatic membrane channels, proteins that interact with plasmatic membrane lipids.

9. The method according to claim 6, wherein said cellular membrane receptor is selected from the group which comprises receptors coupled with G proteins, receptors with an enzymatic activity, channel receptors.

10. The method according to any of the previous claims, wherein said  $\text{Ca}^{2+}$ -sensitive recombinant protein probe is characterized in that it comprises the amino acidic sequence of at least one  $\text{Ca}^{2+}$ -sensitive photo-protein, or parts thereof.

11. The method according to claim 10, wherein the protein probe additionally comprises a signal sequence and/or the amino acidic sequence of a cellular effector.

12. The method according to claim 11, wherein said cellular effector is selected from the group which consists of ionic channel, regulating protein, cellular membrane receptor.

20 13. The method according to claim 12, wherein said ionic channel is selected from the group which comprises voltage-dependent  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  channel receptors.

14. The method according to claim 12, wherein said regulating protein is selected from the group which comprises 25 protein-kinase, phosphatase, adenylate cyclase, proteins

that link plasmatic membrane receptors, proteins that interact with plasmatic membrane channels, proteins that interact with plasmatic membrane lipids.

15. The method according to claim 14, wherein said protein-kinase are protein-kinase C (PKC).

16. The method according to claim 12, wherein said cellular membrane receptor is selected from the group which comprises receptors coupled with G proteins, receptors with an enzymatic activity, channel receptors.

10 17. The method according to claims 10 and 11, wherein said signal sequence directs the Ca<sup>2+</sup>-sensitive photoprotein, preferably aequorin, to a cellular compartment.

18. The method according to any of the claims from 10 to 17, wherein the protein probe is a condensation protein selected from the group which consists of PKC-aequorin (PKC-AEQ), shc-aequorin (shc-AEQ), SNAP-aequorin (SNAP-AEQ), mt-aequorin (mt-AEQ), cytosol aequorin (cyt-AEQ).

19. The method according to claim 18, wherein the PKC-aequorin is selected from the group which comprises PKC beta-aequorin, PKC delta-aequorin, PKC epsilon-aequorin, PKC zeta-aequorin, PKC gamma-aequorin, PKC alpha-aequorin, PKC-lambda-aequorin, PKC theta-aequorin, PKC eta-aequorin.

20. The method according to claim 18, wherein the shc-aequorin is selected from the group consisting of p66shc-

aequorin, p46shc-aequorin, p52shc-aequorin.

21. The method according to claim 1, wherein the expression vector of phase a) is a eukaryotic vector.

22. The method according to claim 1, wherein said at 5 least one mammal cellular line is previously engineered so as to express a heterologic native or chimeric protein.

23. The method according to claim 22, wherein said heterologic protein is selected from the group which consists of a receptor, an enzyme, an ionic channel or a 10 cellular effector.

24. The method according to claim 23, wherein said receptor is a chimeric receptor.

25. The method according to claim 24, wherein said chimeric receptor is characterized in that it has the intra-cellular portion of a receptor coupled with variations in the concentration of calcium and the extra-cellular portion of a receptor coupled with the production of cAMP.

26. The method according to claim 23, wherein said 20 ionic channel is selected from the group which comprises voltage-dependent  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  channel receptors.

27. The method according to claim 23, wherein said 25 cellular effector is selected from the group which consists of ionic channel, regulating protein, cellular membrane receptor.

28. The method according to claim 27, wherein said ionic channel is selected from the group which comprises voltage-dependent  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  channel receptors.

29. The method according to claim 27, wherein said regulation protein is selected from the group which comprises protein-kinase, phosphatase, adenylate cyclase, proteins that link plasmatic membrane receptors, proteins that interact with plasmatic membrane channels, proteins that interact with plasmatic membrane lipids.

10 30. The method according to claim 27, wherein said cellular membrane receptor is selected from the group which comprises receptors coupled with G proteins, receptors with an enzymatic activity, channel receptors.

15 31. A  $\text{Ca}^{2+}$ -sensitive recombinant protein probe, characterized in that it comprises amino acidic sequences of at least one  $\text{Ca}^{2+}$ -sensitive photo-protein, or parts thereof, and a cellular effector or a signal sequence, condensed together.

20 32. The probe according to claim 31, wherein said  $\text{Ca}^{2+}$ -sensitive photo-protein is aequorin.

33. The probe according to claims 31 and 32, wherein said protein probe additionally comprises a signal sequence and/or the amino acidic sequence of a cellular effector.

25 34. The probe according to claims 31 to 33, wherein said

cellular effector is selected from the group which consists of ionic channel, regulating protein, cellular membrane receptor.

35. The probe according to claim 34, wherein said ionic channel is selected from the group which comprises voltage-dependent  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  channel receptors.

36. The probe according to claim 34, wherein said regulating protein is selected from the group which comprises protein-kinase, phosphatase, adenylate cyclase, proteins that link plasmatic membrane receptors, proteins that interact with plasmatic membrane channels, proteins that interact with plasmatic membrane lipids.

37. The probe according to claim 36, wherein said protein-kinase are protein kinase C (PKC).

38. The probe according to any of the claims from 31 to 37, wherein said probe is a condensation protein which consists of PKC-aequorin (PKC-AEQ).

39. The probe according to claim 38, wherein the PKC-aequorin is selected from the group which comprises PKC beta-aequorin (PCK beta: rif. M13975), PKC delta-aequorin (PCK delta: rif. M18330), PKC epsilon-aequorin (PCK epsilon: rif. AF028009), PKC zeta-aequorin (PCK zeta: rif. M18332), PKC gamma-aequorin, PKC alpha-aequorin (PCK alfa: rif. M13973), PKC-lambda-aequorin, PKC theta-aequorin (PCK theta: rif. L07032), PKC eta-aequorin.

40. The probe according to claim 36, wherein said proteins which link plasmatic membrane receptors belong to the shc family.

41. The probe according to claim 40, wherein the protein  
5 is selected from the group comprising p46shc, p52shc and p66shc.

42. The probe according to claim 34, wherein said cellular membrane receptor is selected from the group which comprises receptors coupled with G proteins, receptors  
10 with an enzymatic activity, channel receptors.

43. The probe according to claims 31 to 42, wherein said signal sequence directs the  $\text{Ca}^{2+}$ -sensitive photo-protein, preferably aequorin, towards a cellular compartment.

44. Use of the  $\text{Ca}^{2+}$ -sensitive recombinant probe as defined in claims 31 to 42, for the screening of molecules capable of generating the alteration of an intracellular parameter, said alteration being converted into a proportional variation in the intracellular concentration of the  $\text{Ca}^{2+}$  ion.  
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45. Use according to claim 44, wherein said alteration of an intracellular parameter is selected from the group which comprises variation in concentration of a second messenger, translocation to the membrane or activation/inactivation state of a cellular effector.  
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46. Use according to claim 45, wherein said second mes-  
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senger is selected from the group which comprises cyclic nucleotides, nucleotides of adenosine, diacyl glycerol,  $\text{Ca}^{2+}$  and inositol 1,4,5 triphosphate.

47. Use according to claim 45, wherein said cellular effector is selected from the group which consists of ionic channel, regulating protein, cellular membrane receptor.

48. Use according to claim 47, wherein said ionic channel is selected from the group which comprises voltage-dependent  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  channel receptors.

10 49. Use according to claim 47, wherein said regulation protein is selected from the group which comprises protein-kinase, phosphatase, adenylate cyclase, proteins that link plasmatic membrane receptors, proteins that interact with plasmatic membrane channels, proteins that 15 interact with plasmatic membrane lipids.

50. Use according to claim 47, wherein said cellular membrane receptor is selected from the group which comprises receptors coupled with G proteins, receptors with an enzymatic activity, channel receptors.